

Keratinocyte Adherens Junctions Initiate Nuclear Signaling by Translocation of Plakoglobin from the Membrane to the Nucleus

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Because changes in cell–cell adhesion have profound effects on cellular behavior, we hypothesized a link between the adhesion and signaling functions of plakoglobin and β -catenin. To investigate the existence of adherens-junction-mediated signaling, we used peroxovanadate to tyrosine phosphorylate plakoglobin and β -catenin and to dissociate adherens junctions. The distribution of plakoglobin and β -catenin was determined by immunofluorescence, western blot analysis, pulse-chase radiolabeling, and biochemical subcellular fractionation. Coimmunoprecipitation studies from nuclear fractions, gel-shift assays, and transient transfections with T cell factor (TCF)/lymphoid enhancer factor (LEF) optimized promoter reporter constructs were used to investigate the ability of plakoglobin and β -catenin that had redistributed from the membrane to the

nucleus to form functional transcriptional regulatory complexes with TCF/LEF family member transcription factors. Tyrosine phosphorylation of plakoglobin and β -catenin resulted in their rapid translocation from the cell membrane to the nucleus. Nuclear translocation was associated with increased plakoglobin and decreased β -catenin binding to nuclear TCF/LEF and downregulation of gene transcription from TCF/LEF reporter constructs. These results are consistent with a signaling pathway initiated by structural changes in the adherens junction in which adherens-junction-derived plakoglobin regulates nuclear transcription by antagonizing the binding of β -catenin to TCF/LEF proteins. *Key words:* adherens junction/ β -catenin/phosphorylation/plakoglobin/signal transduction. *J Invest Dermatol* 121:242–251, 2003

In addition to their structural role in mediating adhesion, both cell–substrate and cell–cell adhesion complexes are thought to have functional roles as signaling proteins. For example, changes in integrin-mediated adhesion activate intracellular signaling cascades (for review see Giancotti and Ruoslahti, 1999; Dedhar, 2000). Along with desmosomes and tight junctions, adherens junctions function in epithelial cell–cell adhesion. In human keratinocytes, as in other epithelia, components of the adherens junction include cadherins (Takeichi, 1988; Takeichi, 1991), β -catenin or plakoglobin, and α -catenin (Ozawa *et al*, 1989; Nagafuchi *et al*, 1991; Butz and Kemler, 1994). Recent studies have suggested that adherens junctions might also function in signal transduction. Homophilic binding of the extracellular domain of cadherin has been shown to inhibit intracellular RhoA GTPase activity and increase intracellular Rac1 GTPase activity (Noren *et al*, 2001). In tissue culture, overexpression of cadherin can downregulate the signaling function of β -catenin by binding and sequestering β -catenin at the membrane (Sadot *et al*, 1998; Simcha *et al*, 1998; Orsulic *et al*, 1999; Stockinger *et al*, 2001).

In addition to its structural role in mediating cell–cell adhesion, β -catenin also functions in Wnt-mediated signal transduction (Funayama *et al*, 1995; Miller and Moon, 1996). The cytoplasmic and nuclear levels of β -catenin are typically low due to proteasome-mediated degradation (Aberle *et al*, 1997), which requires the formation of a complex consisting of β -catenin, the tumor suppressor protein adenomatous polyposis coli (Rubinfeld *et al*, 1993; Su *et al*, 1993; Munemitsu *et al*, 1995), the serine/threonine kinase glycogen synthetase kinase 3 (GSK-3) (Rubinfeld *et al*, 1996; Yost *et al*, 1996), and axin (Zeng *et al*, 1997; Ikeda *et al*, 1998). Wnt proteins, a family of secreted polypeptides, bind to transmembrane frizzled receptors (Wang *et al*, 1996; He *et al*, 1997) on cell membranes to initiate a signal transduction cascade that, through the actions of dishevelled, stabilizes cytoplasmic β -catenin. Consequently, increased levels of β -catenin result in formation of bipartite transcription complexes between β -catenin and the T cell factor (TCF)/lymphoid enhancer factor (LEF) family transcription factors (Behrens *et al*, 1996; Molenaar *et al*, 1996; van de Wetering *et al*, 1997). The subsequent binding of nuclear-localized TCF/ β -catenin complexes to DNA containing specific consensus sequences recognized by TCF/LEF family members activates transcription of target genes including c-Myc (He *et al*, 1998) and cyclin D1 (Tetsu and McCormick, 1999). In keratinocyte epithelial biology, Wnt signaling has been implicated in hair follicle development (van Genderen *et al*, 1994; Zhou *et al*, 1995; DasGupta and Fuchs, 1999; Millar *et al*, 1999; Huelsken *et al*, 2001; Merrill *et al*, 2001; Andl *et al*, 2002; Li *et al*, 2002) and in maintenance of proliferative potential (Zhu and Watt, 1999).

Like β -catenin, plakoglobin binds to TCF/LEF proteins; however, plakoglobin's signaling function is less clearly defined. Because of their structural similarities, plakoglobin has been

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Abbreviations: E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]-agmatine; GSK, glycogen synthase kinase; LEF, lymphoid enhancer factor; PARP, poly ADP ribose polymerase; TCF, T cell factor; Wg, wingless.

thought to function like β -catenin to activate transcription from TCF/LEF response elements; however, in contrast to β -catenin, which forms a ternary complex with LEF/TCF and DNA, Zhurinsky and colleagues have shown that neither plakoglobin–TCF4 nor plakoglobin–LEF1 binary complexes bound DNA containing the optimized TCF/LEF promoter (Zhurinsky *et al*, 2000). In cells transfected with plakoglobin cDNA, expression of plakoglobin has been observed to increase the levels of nuclear and cytoplasmic β -catenin (Simcha *et al*, 1998; Williams *et al*, 2000). In particular, membrane-anchored plakoglobin constructs, which are themselves restricted from entry into the nuclei, increase nuclear and cytoplasmic β -catenin levels by saturating the degradative machinery and displacing β -catenin from the proteasome targeting and degradation complexes (Miller and Moon, 1997). Increased transcription from TCF reporter constructs that has been observed in cells induced to overexpress plakoglobin is probably due to the displacement of β -catenin by plakoglobin from multiprotein cellular complexes; the displaced β -catenin is then available for binding to TCF/LEF proteins with the resultant formation of active transcriptional complexes. Recently, formation of a ternary complex between β -catenin–TCF4 and plakoglobin has been described in which plakoglobin inhibits the transcriptional activity of β -catenin–TCF4 (Miravet *et al*, 2002).

The cell–cell adhesion and signaling functions of plakoglobin and β -catenin are generally thought to be independent because a direct link between their adhesion and signaling functions has not yet been demonstrated. Although several studies have reported nuclear accumulation of catenins associated with catenin phosphorylation or disruption of cell–cell adhesion (Muller *et al*, 1999; Eger *et al*, 2000; Danilkovitch-Miagkova *et al*, 2001), none has conclusively demonstrated that nuclear catenin is derived from the membrane and not a result of new protein synthesis and/or stabilization of cytoplasmic and nuclear catenin pools by downregulation of proteasome-mediated degradation. In human keratinocytes, tyrosine phosphorylation of plakoglobin and β -catenin disrupts cell–cell adhesion and also disrupts the association of these proteins with E-cadherin and α -catenin (Hu *et al*, 2001), raising the possibility that β -catenin and plakoglobin released from the adherens junction move to the nucleus to exert a signaling function independent of Wnt binding to transmembrane frizzled receptors. Because *in vitro* data demonstrate a low affinity of plakoglobin–TCF/LEF complexes for TCF/LEF promoters (Zhurinsky *et al*, 2000), the effect of a coincident increase in nuclear β -catenin and nuclear plakoglobin, such as occurs upon dissociation of keratinocyte adherens junctions, might be to either activate or repress transcription from TCF/LEF promoters.

In this report we demonstrate that tyrosine phosphorylation of cell-membrane-associated plakoglobin results in (1) direct movement (e.g., translocation) of plakoglobin from the cell membrane to the nucleus, (2) increased nuclear localization of plakoglobin and increased nuclear levels of complexes between TCF/LEF family member transcription factors and plakoglobin, and (3) inhibition of transcription from TCF/LEF reporter constructs. Furthermore, nuclear coimmunoprecipitation studies suggest that transcriptional inhibition by plakoglobin results from competition between nuclear-localized plakoglobin and β -catenin for TCF/LEF proteins in which the binding of plakoglobin to TCF/LEF proteins displaces β -catenin. These findings suggest a direct signaling pathway from the adherens junction to the nucleus initiated by changes in adherens junction structure and mediated directly by plakoglobin released from the adherens junction by tyrosine phosphorylation.

MATERIALS AND METHODS

Materials Monoclonal antibodies to human β -catenin and plakoglobin (N-terminal), a horseradish peroxidase conjugated monoclonal antiphosphotyrosine antibody (RC20-HRPO conjugate), a pancytokeratin monoclonal antibody (clone AE1/AE3), and Protein G

Sepharose beads were from Zymed (San Francisco, CA). Monoclonal antibodies to human E-cadherin, a C-terminal monoclonal antibody to human plakoglobin, and a monoclonal antibody to poly ADP ribose polymerase (PARP) were from Transduction Laboratories (Lexington, KY). Anti-TCF/LEF (Pan) antibody, clone REMB6, was from Kamiya Biomedical (Seattle, WA). A polyclonal anti-lactate dehydrogenase V antibody was from Cortex Biochem (Sanleandro, CA). For western blot detection, secondary goat antimouse horseradish peroxidase conjugated IgG and enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Buckinghamshire, UK). For immunofluorescent staining, secondary fluorescein isothiocyanate and rhodamine conjugated goat antihuman/rat/mouse IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA). [35 S] methionine/cysteine (1000 Ci per mM) and [α - 32 P] dATP (3000 Ci per mM) were from ICN (Costa Mesa, CA). Electrophoresis reagents were from Bio-Rad (Hercules, CA). Topflash and Fopflash reporter constructs were obtained from Upstate Biotechnology (Lake Placid, NY). A control vector was derived from the Topflash reporter plasmid by excising the TCF/LEF binding fragment (324 bp) with NdeI/SalI and subsequent religation of the vector plasmid. Wingless-conditioned medium was the kind gift of Don McEwen and Mark Peifer (Department of Biology, University of North Carolina – Chapel Hill, NC) (van Leeuwen *et al*, 1994). All other reagents were of standard grade from Sigma (St Louis, MO).

Tissue culture Primary human keratinocytes established from human neonatal foreskins were obtained from Cascade Biologics (Portland, OR) and cultured according to the recommended procedure using NHK 154 medium (0.2 mM CaCl₂) supplemented with human keratinocyte growth supplement (Cascade Biologics) and penicillin, streptomycin, and amphotericin B (Cascade Biologics) according to the manufacturer's recommendations. Cells were expanded for two passages and frozen in 10% dimethylsulfoxide. Third passage keratinocytes were used for all experiments. For immunoprecipitation experiments, cultures were grown on Falcon 100 \times 20 mm polystyrene Petri dishes to 80% confluence. For some experiments, cells were pretreated with LiCl (10 mM) or wingless-conditioned medium for 8 h prior to harvesting or subsequent treatment with peroxovanadate. SW480 colorectal adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to the recommended procedures in Leibovitz's L-15 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Cells were treated with or without peroxovanadate (0.5 mM Na₃VO₄, 3 mM H₂O₂) for 15 min prior to harvesting.

Confocal immunofluorescent microscopy Immunofluorescent staining of human keratinocytes with monoclonal antibodies to plakoglobin and β -catenin was as previously described (Hu *et al*, 2001). Cells were treated with or without peroxovanadate (0.5 mM Na₃VO₄, 3 mM H₂O₂) for 15 min prior to fixation. Images were obtained using a Zeiss laser scanning confocal microscope.

Subcellular fractionation Cell fractions were prepared according to established protocols (Ausubel *et al*, 1995) with minor modifications. Briefly, monolayer cells were quickly washed once with hypotonic buffer (buffer A: 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 10 μ M L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane N-[N-(L-3-transcarboxyirane-2-carbonyl)-L-leucyl]-agmatine (E-64), 100 μ M leupeptin, 10 μ M pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and then were incubated with buffer A on ice for 10 min. The cells were then scraped and the crude cell lysates were dounced in a homogenizer with pestle A for 15 slow strokes and centrifuged at 209g at 4°C for 5 min in an Eppendorf benchtop centrifuge. The supernatants (S1 fraction) were used for further extraction of membrane and cytoplasmic fractions and the pellets (P1 fraction) were used for nuclear fractions as follows.

S1 supernatant The supernatants were carefully transferred into clean tubes and centrifuged at 100,000g at 4°C for 1 h in a Beckman TL100 ultracentrifuge. The supernatant from this centrifugation step was carefully transferred and saved as the cytoplasmic fraction. The pellets were washed with hypotonic buffer twice and resuspended in lysis buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 1.5 mM MgCl₂, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM DTT, 1.0% Nonidet P-40, 10 μ M E-64, 100 μ M leupeptin, 10 μ M pepstatin, and 1 mM PMSF), incubated at 4°C for 1 h with rotating, and then centrifuged at 13,700g 4°C for 15 min to remove the insoluble material. The supernatant was collected and saved as the membrane fraction. The insoluble pellet from this fraction was washed twice with lysis buffer, resuspended by incubation in 8 M urea, 4% CHAPS, 10 mM

Tris-HCl, pH 7.4, with gentle rocking on a nutator for 1 h at 4°C, and centrifuged at 13,700g 4°C for 15 min; the supernatants were saved as the detergent insoluble fraction.

P1 pellets The pellets were washed with hypotonic buffer twice and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 10 μM E-64, 100 μM leupeptin, 10 μM pepstatin, and 1 mM PMSF), incubated on ice for 20 min, then dounced in a homogenizer with pestle B for 15 slow strokes, and centrifuged at 13,700g the benchtop centrifuge at 4°C for 20 min. The supernatant was saved as nuclear fractions. Protein concentrations in all fractions were determined by Bradford assay. The purity of each fraction was followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot detection with antibodies (dilutions) to the transmembrane protein E-cadherin (1:2500), the cytoplasmic enzyme lactate dehydrogenase (1:1000), the nuclear enzyme PARP (1:500), and the cytoskeletal intermediate filament protein keratin (AE1/AE3, 1:500) as markers of the detergent soluble membrane, cytoplasmic, nuclear, and detergent insoluble fractions, respectively.

Immunoprecipitation Normal human keratinocytes or SW480 cells, grown in tissue culture, were treated with or without peroxovanadate (0.5 mM Na₃VO₄, 3 mM H₂O₂), fractionated as described above, and subjected to immunoprecipitation, as previously described (Hu *et al*, 2001), with the following primary antibodies at the indicated concentrations: β-catenin (Zymed, 0.5 μg antibody per 100 μg total protein), C-terminal plakoglobin (Zymed, 0.5 μg antibody per 100 μg total protein), pan TCF/LEF (1 μg antibody per 100 μg extract). Immunoprecipitated proteins were separated by 7.5% SDS-PAGE (Laemmli, 1970), electrotransferred to nitrocellulose, probed with primary antibodies (β-catenin 1:1000; N-terminal plakoglobin 1:2000; antiphosphotyrosine 1:2500; C-terminal plakoglobin 1:2000; TCF/LEF 1:250), and developed with horseradish peroxidase conjugated secondary antibody and ECL reagents (Amersham) as previously described (Hu *et al*, 2001). Horseradish peroxidase conjugated antiphosphotyrosine antibodies were developed directly.

Pulse-chase analysis Keratinocytes were cultured to 80% confluence and incubated with medium containing [³⁵S]-methionine/cysteine overnight at 37°C. Cells were washed once with cold medium and then chased with cold medium for 4 h at 37°C. Cells were then treated with or without peroxovanadate (0.5 mM Na₃VO₄, 3 mM H₂O₂) for 15 min. Cells were fractionated into membrane, cytoplasmic, and nuclear fractions, as described above, and protein concentrations in each fraction were determined by Bradford assay. Fractions containing equal amounts of protein were subjected to immunoprecipitation followed by SDS-PAGE. Quantitation of radioactivity in SDS-PAGE separated proteins was by phosphorimage analysis of dried gels using a Molecular Dynamics Phosphorimager and ImageQuant software.

TCF/LEF reporter assays Keratinocytes and SW480 cells, grown to 80% confluence in 24-well plates, were transfected with 0.4 μg of the Topflash (Korinek *et al*, 1997) or Fopflash reporter or control vectors using the Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were treated with (1) peroxovanadate (0.5 mM Na₃VO₄, 3 mM H₂O₂) for 15 min, (2) 10 mM LiCl for 8 h, (3) 10 mM LiCl for 8 h followed by peroxovanadate for 15 min, (4) wingless-conditioned medium for 8 h, or (5) wingless-conditioned medium for 8 h followed by peroxovanadate for 15 min, or (6) normal medium only for control. Luciferase activity was measured using luciferase assay kits (Promega, Madison, WI) from six parallel wells for each treatment condition; values were read using a Lumat LB9501 luminometer (Berthold, Germany). Activities are expressed as fold stimulation and are related to respective reporter activities obtained with control vector plasmid, which retained the luciferase gene downstream from a basal promoter but from which the TCF/LEF optimized promoter had been excised.

Gel-shift assays Assays were performed as described previously (Korinek *et al*, 1997) using the Amersham Pharmacia Bandshift kit. As the optimal TCF probe, we used a 34-mer double-stranded oligonucleotide AATTACTCTGGTACTGGCCCTTTGATCTTTCTGG. The mutant TCF probe comprised a 34-mer double-stranded oligonucleotide AATTACTCTGGTACTGGCCCTTTCCGGTTTCTGG. Probes were radiolabeled with [α-³²P] dATP and Klenow according to the manufacturer's recommendations. Binding reactions were done with 5 μg nuclear protein, 0.5 ng probe, 1 μg poly(dI-dC), 10% glycerol, 0.05% Nonidet P-40, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM DTT in a total 20 μL reaction volume. The samples were incubated for

20 min at room temperature. For supershift experiments, 0.5 μg of anti-β-catenin or antiplakoglobin antibody (Transduction Laboratories) was added and incubated for another 20 min. The samples were subsequently subjected to 5% nondenaturing polyacrylamide gel electrophoresis.

RESULTS

Tyrosine phosphorylation of plakoglobin and β-catenin results in a rapid redistribution of both plakoglobin and β-catenin from the cell membrane to the nucleus. Keratinocytes incubated with the tyrosine phosphatase inhibitor peroxovanadate demonstrated a rapid change in the distribution of both plakoglobin and β-catenin. Confocal immunofluorescent staining of plakoglobin and β-catenin in untreated keratinocytes was predominantly localized to the cell membrane, whereas a shift in staining to the cytoplasm and nucleus was observed in peroxovanadate-treated keratinocytes (**Fig 1a**). This change was more pronounced for plakoglobin.

Biochemical fractionation of keratinocytes into detergent soluble membrane, cytoplasmic, nuclear, and detergent insoluble cytoskeletal fractions revealed similar results. Compared to control keratinocytes, increased amounts of plakoglobin and β-catenin were detected by western blot analysis in the cytoplasmic and nuclear fractions of peroxovanadate-treated keratinocytes (**Fig 1b**). The lack of detectable E-cadherin, lactate dehydrogenase, and keratin by western blot analysis of nuclear fractions demonstrated that nuclear fractions were not contaminated with membrane, cytoplasmic, or cytoskeletal proteins, respectively. The enzyme PARP, a marker for the nuclear compartment, was detected only in nuclear extracts. Of note, cleavage of PARP, an apoptosis-associated event, was observed in treated keratinocytes. Additionally, the SDS-PAGE migration of plakoglobin and β-catenin from peroxovanadate-treated fractions was reduced, consistent with their post-translational modification by phosphorylation. Although not shown, when the ECL reaction was allowed to continue so that the film became overdeveloped, a small fraction of nontyrosine phosphorylated plakoglobin and β-catenin, probably less than 5% of the nuclear pool, could be detected.

In contrast to its ability to induce dissociation of plakoglobin from adherens junctions (Hu *et al*, 2001), peroxovanadate-mediated tyrosine phosphorylation of plakoglobin did not dissociate plakoglobin from desmosomes. Unlike adherens junctions, desmosomes are detergent insoluble. A marked reduction in plakoglobin and β-catenin was observed in the detergent soluble membrane fraction of peroxovanadate-treated cells; however, using the detergent insoluble fraction as a marker for desmosomes (Pasdar and Nelson, 1988; 1989; Pasdar *et al*, 1995), we observed no change between the amount of plakoglobin immunoreactivity detected on western blots in the insoluble fraction from control and from peroxovanadate-treated keratinocytes (**Fig 1b**).

Immunoprecipitation of plakoglobin and β-catenin from nuclear fractions demonstrated increased levels of plakoglobin and β-catenin in nuclei of peroxovanadate-treated cells compared to controls. Additionally, the predominant isoforms of nuclear plakoglobin and β-catenin immunoprecipitated from peroxovanadate-treated cells were tyrosine phosphorylated as revealed by immunoblotting with antiphosphotyrosine antibodies (**Fig 1c**).

The membrane is the source of nuclear plakoglobin. The increased amounts of nuclear-localized plakoglobin observed after peroxovanadate treatment could be due to (1) release of plakoglobin from cell-membrane-localized adherens junctions, (2) increased protein synthesis, or (3) stabilization of cytoplasmic plakoglobin by downregulation of cellular degradative pathways. Pulse-chase labeling experiments were utilized to differentiate between these alternatives. Pulse-chase labeling enabled selective labeling of junction-associated plakoglobin as nonjunction-associated protein in the cytoplasm and nucleus was rapidly

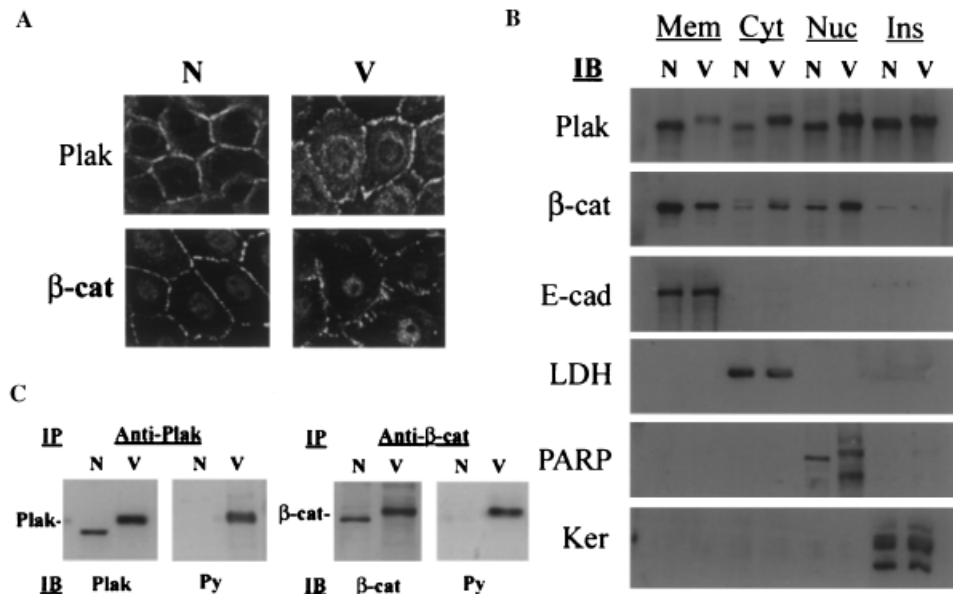


Figure 1. Tyrosine phosphorylation of plakoglobin and β -catenin shifts their distribution from the membrane to the cytoplasm and nucleus.

(A) Confocal immunofluorescent staining of cultured normal human keratinocytes for β -catenin or plakoglobin. Control cells (N) or cells treated with peroxovanadate (V) for 15 min were fixed and immunohistochemically stained with monoclonal antibodies to β -catenin (β -cat) and plakoglobin (Plak) and examined by confocal immunofluorescent microscopy. (B) Subcellular fractionation. Control cells (N) or cells treated with peroxovanadate (V) for 15 min were separated into detergent soluble membrane (Mem), cytoplasmic (Cyt), nuclear (Nuc), and detergent insoluble (Ins) fractions as described in *Materials and Methods*. Ten micrograms from each fraction were separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to plakoglobin (Plak), β -catenin (β -cat), E-cadherin (E-cad), lactate dehydrogenase V (LDH), poly ADP ribose polymerase (PARP), and pan-cytokeratin (Ker). Increased levels of plakoglobin and β -catenin were detected in the cytoplasmic and nuclear fractions of peroxovanadate-treated cells. (C) Immunoprecipitation from nuclear fractions. Nuclear extracts from control cells (N) or cells treated with peroxovanadate (V) for 15 min were immunoprecipitated with antibodies to plakoglobin (IP: Anti-Plak) and β -catenin (IP: Anti- β -cat) followed by immunoblot detection with antibodies to plakoglobin (IB: Plak) and β -catenin (IB: β -cat), respectively. Blots were stripped and reprobed with antiphosphotyrosine antibodies (IB: Py).

degraded; whereas, junction-associated protein persisted. After incubation in the presence of medium containing [35 S]-methionine and [35 S]-cysteine, 35 S-labeled plakoglobin was detected in membrane, cytoplasmic, and nuclear fractions (Fig 2, lane 1). After being chased with cold medium for 4 h, there was a differential decrease in the levels of 35 S-plakoglobin from each of these three fractions, reflective of the relative stability of plakoglobin in these different cellular compartments. Cytoplasmic and nuclear plakoglobin fractions were less stable than the membrane-associated plakoglobin fraction; the cytoplasmic and nuclear fractions represent 18.8% and 23.4% of the initial counts of these fractions, respectively, whereas the membrane fraction decreased to 63% of the initial counts (Fig 2, lane 2).

Subsequent treatment of 35 S pulse-chase-labeled keratinocytes with peroxovanadate resulted in redistribution of 35 S-labeled plakoglobin from the membrane to the nucleus (Fig 2, lane 3). Relative to the band density prior to peroxovanadate treatment (Fig 2, lane 2), the density of membrane 35 S-plakoglobin decreased to 40% (lane 3 divided by lane 2); the cytoplasmic fraction showed a modest increase (125%); however, the nuclear fraction of 35 S-labeled plakoglobin showed an almost 4-fold increase (375%). Because it is not due to new protein synthesis, the increase in nuclear plakoglobin is probably due to movement into the nucleus of plakoglobin released from the membrane.

The translocation of plakoglobin from the membrane to the nucleus results in the binding of nuclear plakoglobin to TCF/LEF family members. Coimmunoprecipitation experiments from nuclear extracts were used to determine if the observed nuclear translocation resulted in complex formation between TCF/LEF transcription factors and either plakoglobin or β -catenin. After peroxovanadate treatment, increased levels of both plakoglobin and β -catenin were detected in nuclear extracts; the increase was more pronounced for plakoglobin (Fig 3a, N, V).

To enhance the catenin signal, keratinocytes were incubated in the presence of LiCl prior to exposure to peroxovanadate. By inhibiting the serine/threonine kinase GSK-3, LiCl disrupts the degradation of cytoplasmic β -catenin, allowing increased levels of cytoplasmic and nuclear β -catenin to accumulate in the absence of Wnt signaling (Klein and Melton, 1996; Stambolic *et al*, 1996). Consistent with the effects of LiCl in other cell types (Hedgpeth *et al*, 1997), treating keratinocytes with LiCl resulted in increased levels of nuclear plakoglobin and β -catenin (Fig 3a, L). When keratinocytes that had been treated with LiCl were subsequently treated with peroxovanadate for 15 min, nuclear plakoglobin and β -catenin were markedly increased (Fig 3a, L + V); again, the increase was more pronounced for plakoglobin.

To investigate the association of nuclear plakoglobin and β -catenin with TCF/LEF transcription factors, a pan-TCF/LEF antibody was used to immunoprecipitate TCF/LEF family members from keratinocyte nuclear extracts and the immunoprecipitates were probed for the presence of plakoglobin and β -catenin by immunoblotting. Both plakoglobin and β -catenin coimmunoprecipitated with TCF/LEF from nuclear extracts (Fig 3b, N). Compared to nontreated control cells, more plakoglobin coimmunoprecipitated with TCF/LEF from nuclear extracts of cells treated with peroxovanadate; β -catenin similarly coimmunoprecipitated with TCF/LEF from nuclear extracts of both control and peroxovanadate-treated cells. Furthermore, more plakoglobin and β -catenin coimmunoprecipitated with TCF/LEF from nuclei of LiCl-treated cells compared to controls. Relative to cells treated with LiCl alone, however, in keratinocytes treated first with LiCl and then peroxovanadate, the amount of β -catenin coimmunoprecipitating with nuclear TCF/LEF decreased, whereas the amount of plakoglobin coimmunoprecipitating with nuclear TCF/LEF increased (Fig 3b). This reciprocal relationship between the ability of β -catenin and plakoglobin to coimmunoprecipitate

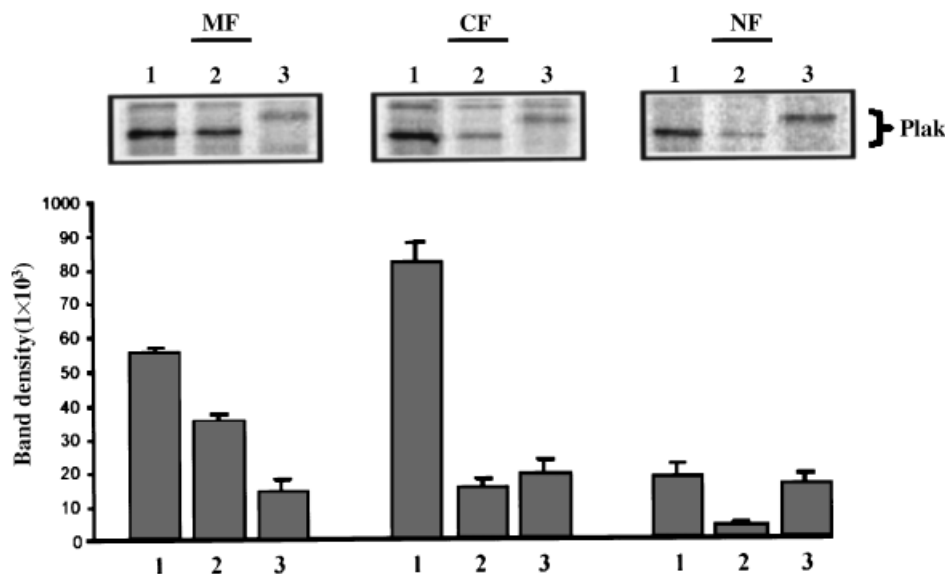


Figure 2. The membrane is the source of nuclear plakoglobin. Keratinocytes were metabolically labeled with ³⁵S methionine/cysteine overnight (1), chased with cold medium for 4 h (2), and then treated with peroxovanadate (3) for 15 min. Cells were fractionated into detergent soluble membrane (MF), cytoplasmic (CF), and nuclear fractions (NF). Plakoglobin was immunoprecipitated from 200 µg of each extract and separated by SDS-PAGE, and the radioactivity in the plakoglobin band was quantitated by phosphoimage analysis. Each bar represents the average of three independent experiments; SEM is shown by error bars. A representative phosphoimage scan of one such experiment is shown in the top panels. After incubation in the presence of medium containing ³⁵S-methionine and ³⁵S-cysteine, ³⁵S-labeled plakoglobin was detected in membrane, cytoplasmic, and nuclear fractions. After being chased with cold medium for 4 h, there is a differential decrease in the levels of ³⁵S-plakoglobin from each of these three fractions reflective of the relative stability/turnover rate of plakoglobin in these different cellular compartments. Cytoplasmic and nuclear plakoglobin fractions are less stable than the membrane-associated plakoglobin fraction. The cytoplasmic and nuclear fractions represent 18.8% and 23.4% of the initial counts of these fractions, respectively, whereas the membrane fraction decreased to 63% of the initial counts (lane 1, ³⁵S-plakoglobin after overnight labeling; lane 2, ³⁵S-plakoglobin after overnight labeling followed by 4 h chase with cold medium; lane 3, ³⁵S-plakoglobin after overnight labeling followed by 4 h chase with cold medium and then 15 min incubation with peroxovanadate; MF, membrane fraction; CF, cytoplasmic fraction; NF, nuclear fraction). Subsequent treatment of ³⁵S pulse-chase labeled keratinocytes with peroxovanadate resulted in redistribution of ³⁵S-labeled plakoglobin from the membrane to the nucleus (compare lanes 2 and 3). Relative to the band density prior to peroxovanadate treatment, the density of membrane ³⁵S-plakoglobin decreased to 40% (lane 3 divided by lane 2), and the cytoplasmic fraction showed a modest increase (125%); however, the nuclear fraction of ³⁵S-labeled plakoglobin showed almost a 4-fold increase (375%) demonstrating that the increase in nuclear plakoglobin in peroxovanadate-treated cells is due to a redistribution of membrane-associated plakoglobin to the nucleus.

with TCF/LEF proteins from nuclear extracts suggests a competition between plakoglobin and β -catenin for binding to TCF/LEF proteins in which plakoglobin binding to TCF/LEF might function to antagonize β -catenin-mediated transcriptional activation. If the displacement of β -catenin from TCF were due to a nonplakoglobin-related consequence of elevated tyrosine phosphorylation, decreased β -catenin binding to TCF/LEF would be expected in SW480 cells, which express large amounts of nuclear and cytoplasmic β -catenin. No decrease in binding of nuclear β -catenin to TCF/LEF was observed in SW480 cells treated with peroxovanadate, LiCl, or LiCl and peroxovanadate (Fig 3c), indicating that the competition observed in keratinocytes is not the result of a nonplakoglobin-related consequence of elevated tyrosine phosphorylation.

When coimmunoprecipitated with TCF/LEF from nuclear extracts of keratinocytes treated with LiCl and peroxovanadate, plakoglobin immunoreactivity migrated on SDS-PAGE as a doublet, suggesting that both tyrosine phosphorylated and non-tyrosine phosphorylated plakoglobin bind to TCF/LEF family members. Additionally, in these samples TCF/LEF coimmunoprecipitated tyrosine and nontyrosine phosphorylated plakoglobin in roughly equimolar concentrations. The observation that the amount of nontyrosine phosphorylated plakoglobin in this nuclear pool was significantly less than the amount of tyrosine phosphorylated plakoglobin suggested an increased affinity of nontyrosine phosphorylated plakoglobin for TCF/LEF. The ability to visualize this species in the LiCl + peroxovanadate treated samples, but not in the peroxovanadate only treated samples was

probably due to the turnover of these proteins in the absence of LiCl. Because of the dynamic equilibrium between cytoplasmic and nuclear protein, both phosphorylated and nonphosphorylated protein are turned over. As there is much more tyrosine phosphorylated protein, in the absence of LiCl the experiment is biased towards detecting this species. When the degradation of plakoglobin is decreased by LiCl, the less prevalent nonphosphorylated species with the greater affinity for TCF/LEF proteins was visualized. These observations indicate that the effect of peroxovanadate-mediated tyrosine phosphorylation of plakoglobin is due to its ability to induce the redistribution of plakoglobin from the adherens junction to the nucleus and that the increased amount of nuclear plakoglobin, and not tyrosine phosphorylation itself, is responsible for plakoglobin's antagonism of β -catenin.

DNA binding catenin–TCF/LEF complexes are not detected in nuclear extracts from peroxovanadate-treated keratinocytes. Gel-shift experiments using the canonical TCF binding site as a probe were used to assay for the presence of DNA binding complexes in nuclear extracts. SW480 cells were used as controls to check the specificity of the DNA probes. As expected from previous reports (Korinek *et al*, 1997; Morin *et al*, 1997), a shifted band was detected in SW480 nuclear extracts when the TCF promoter sequence was used as a probe (Fig 4a, lane 1); the signal from this band was markedly diminished when extracts were incubated with the mutated probe demonstrating specificity (Fig 4a, lane 2). Additionally, β -catenin antibodies caused a supershift in the TCF probe (Fig 4a, lane 3)

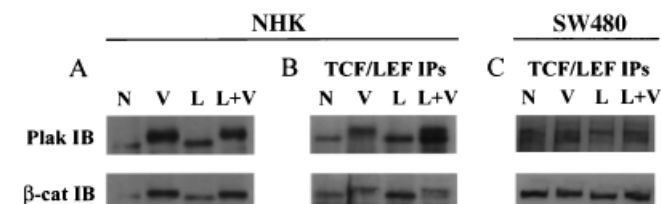


Figure 3. Binding of nuclear plakoglobin and β -catenin to TCF/LEF proteins. (A) Immunoblot detection of β -catenin and plakoglobin in nuclear extracts of keratinocytes. Ten micrograms of nuclear extract from control (N), peroxovanadate-treated (V), LiCl-treated (L), and LiCl followed by peroxovanadate treated (L + V) keratinocytes separated by SDS-PAGE and immunoblotted with antibodies to β -catenin (β -cat IB) or plakoglobin (Plak IB). Increased levels of plakoglobin and β -catenin were detected in nuclear extracts of peroxovanadate-treated keratinocytes compared to controls. The migration of plakoglobin and β -catenin from peroxovanadate-treated extracts is retarded consistent with tyrosine phosphorylation. Increased amounts of plakoglobin and β -catenin were detected in nuclear extracts of cells treated with LiCl; however, no retardation in their SDS-PAGE migration was observed. Exposure of keratinocytes to LiCl and subsequent treatment with peroxovanadate results in elevated nuclear levels of plakoglobin and β -catenin and their migration is retarded. (B) TCF/LEF immunoprecipitations from human keratinocytes. Two hundred micrograms of nuclear extracts from control (N), peroxovanadate-treated (V), LiCl-treated (L), and LiCl followed by peroxovanadate treated (L + V) keratinocytes were subjected to immunoprecipitation with a pan-TCF/LEF specific monoclonal antibody, separated by SDS-PAGE, and immunoblotted with antibodies to plakoglobin (Plak IB) and β -catenin (β -cat IB). Both plakoglobin and β -catenin coimmunoprecipitated with TCF/LEF from nuclear extracts of control (N) and peroxovanadate (V) treated keratinocytes. More plakoglobin and β -catenin coimmunoprecipitated with TCF/LEF from nuclear extracts of cells pretreated with LiCl (L). Compared to cells treated with only LiCl (L), more plakoglobin and less β -catenin coimmunoprecipitated with TCF/LEF from nuclear extracts of keratinocytes treated with LiCl followed by peroxovanadate (L + V). (C) TCF/LEF immunoprecipitations from SW480 cells. TCF/LEF immunoprecipitations from nuclear extracts of SW480 cells from control (N), peroxovanadate-treated (V), LiCl-treated (L), and LiCl followed by peroxovanadate treated (L + V) cells were immunoblotted with antibodies to plakoglobin (Plak IB) and β -catenin (β -cat IB). In contrast to keratinocytes, no reciprocal relationship is observed in plakoglobin's and β -catenin's association with nuclear TCF/LEF proteins in SW480 cells treated with LiCl and LiCl/peroxovanadate.

indicating the presence of β -catenin in the DNA binding complex.

Examination of keratinocyte nuclear extracts revealed the presence of a DNA binding protein in nuclei of untreated controls (Fig 4b, lane 1). Specificity for TCF binding sites was demonstrated by the decreased signal when the Fopflash mutant sequence probe was used (data not shown). This shifted band was not detected in nuclear extracts of peroxovanadate-treated keratinocytes (Fig 4b, lane 2). Supershift experiments with β -catenin and plakoglobin antibodies failed to identify either of these proteins in complex with DNA in nuclear extracts of control keratinocytes (Fig 4b; lanes 3, 5, respectively). The TCF promoter DNA binding protein in nuclear extracts from control keratinocytes may represent a repressor or, alternatively, it may represent β -catenin-TCF/LEF complexes, and the inability to detect a supershift with β -catenin antibodies may reflect the low levels of β -catenin-probe complexes in these extracts. Decreased binding to DNA was observed with peroxovanadate (Fig 4b, lane 2); however, no supershifts in nuclear extracts from peroxovanadate-treated keratinocytes were seen with either β -catenin or plakoglobin antibodies (Fig 4b; lanes 4, 6, respectively) as would be expected (1) if plakoglobin-TCF/LEF complexes have low affinity for TCF reporter elements or (2) if plakoglobin competes with and displaces the binding of β -catenin to TCF/LEF proteins.

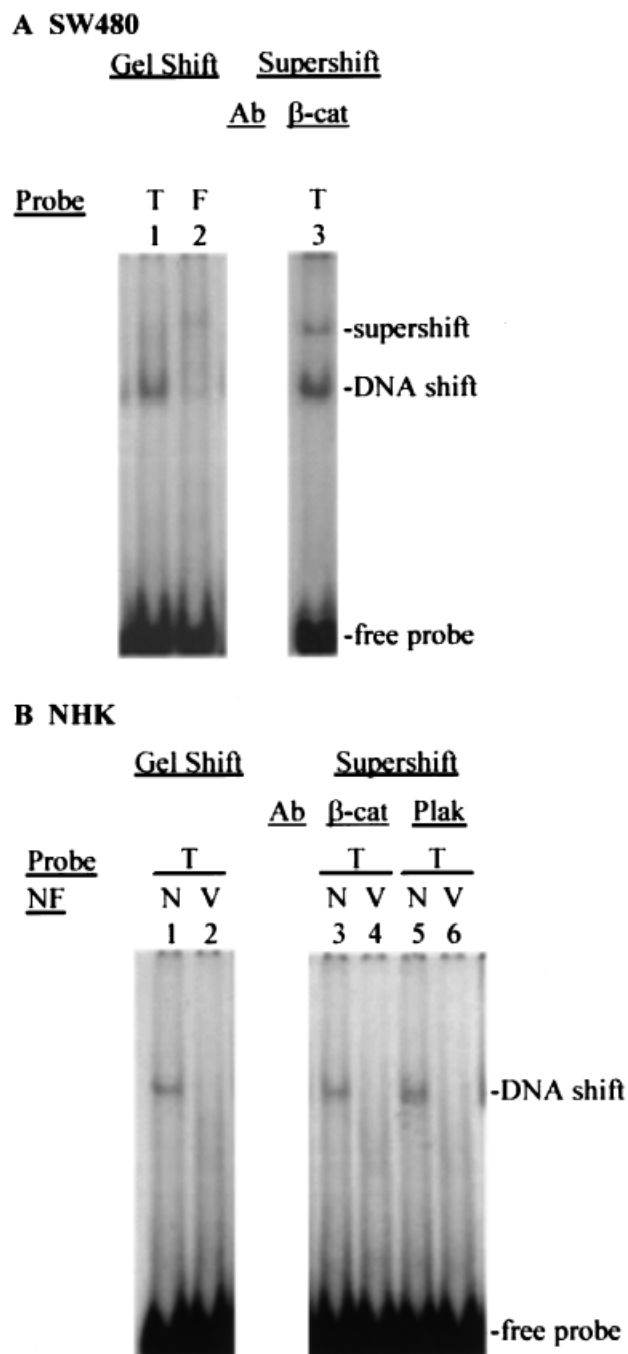


Figure 4. Gel-shift assays. Gel-shift assays were performed using nuclear extracts from nontreated cells (N) or cells pretreated with peroxovanadate for 15 min (V); DNA probes were either the 34-mer double stranded DNA containing the optimized TCF/LEF binding site (T) or the mutated binding site (F) as a control for specific binding. For supershifts, 0.5 μ g β -catenin antibody was added to the samples in lane 3, panel A, and lanes 3–6, panel B. (A) SW480 cells. A shifted band is seen when nuclear extracts from SW480 cells were incubated with the TCF/LEF promoter sequence (T, lane 1), but not with the control mutated promoter (F, lane 2). When incubated with β -catenin antibody, this band was supershifted (T, lane 3) indicating the presence of DNA binding β -catenin-TCF/LEF complexes in nuclear extracts of SW480 cells. (B) Keratinocytes. In nontreated keratinocytes (lane 1, N), a shifted band was identified using the probe containing the optimized TCF/LEF binding site; this band was not seen when keratinocytes were treated with peroxovanadate (lane 2, V). No supershift was seen when nuclear extracts were first incubated with either β -catenin (lanes 3, 4) or plakoglobin (lanes 5, 6) antibodies, indicating the absence of detectable β -catenin-TCF/LEF-DNA and plakoglobin-TCF/LEF-DNA complexes in either control (N) or peroxovanadate-treated (V) keratinocytes.

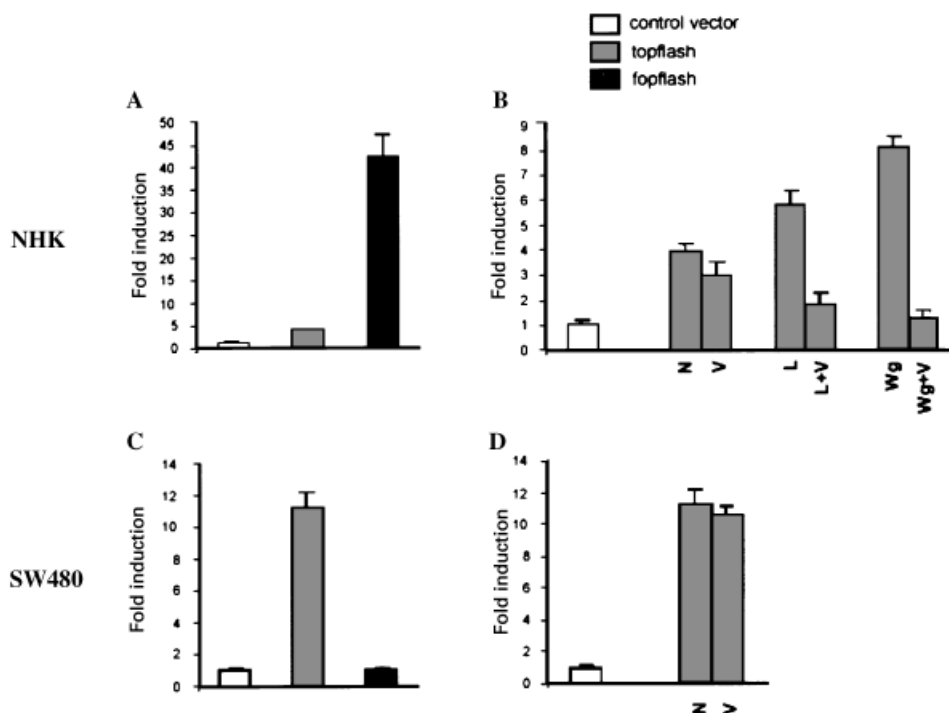


Figure 5. Changes in transcriptional activity from TCF/LEF reporter constructs. Normal human keratinocytes (NHK) and the human colorectal carcinoma cell line SW480 were transiently transfected with the Topflash reporter plasmid containing the TCF/LEF optimized promoter, the Fopflash control plasmid containing a mutated TCF/LEF promoter upstream of the luciferase gene, or a control plasmid that retained the luciferase gene downstream from a basal promoter but from which the TCF/LEF optimized promoter had been excised (control vector). Each bar represents the average \pm standard deviation of three independent experiments. (A) The amount of luciferase activity detected in normal human keratinocytes transfected with pTopflash was approximately 10% of the activity detected when keratinocytes were transfected with the mutated control plasmid pFopflash, suggesting the presence in keratinocytes of a repressor that binds with greater affinity to the optimized TCF/LEF promoter in pTopflash than to the mutated promoter of the control pFopflash. (B) After treatment with peroxovanadate for 15 min (V), no increase in luciferase activity was observed compared to control keratinocytes (N). Incubation of keratinocytes in the presence of LiCl resulted in increased luciferase activity (L). Subsequent peroxovanadate treatment of LiCl-treated keratinocytes resulted in a more pronounced transcriptional repression than was observed for cells that had not been pretreated with LiCl; keratinocytes pretreated with LiCl and then subsequently treated with peroxovanadate for 15 min demonstrated a 4-fold reduction in luciferase activity (L + V). (C) SW480 human colorectal carcinoma cells transfected with either pTopflash, pFopflash, or control plasmid demonstrated the behavior expected for this cell line in which β -catenin's signaling function is constitutively active. Luciferase activity from pTopflash was 10-fold higher than from pFopflash. (D) No significant difference in pTopflash reporter activity was seen between SW480 cells in the presence (V) or absence (N) of peroxovanadate, demonstrating that tyrosine phosphorylation does not directly alter transcriptional activation by β -catenin–TCF/LEF complexes or other downstream components including the transcriptional and translational machinery.

A repressor of TCF/LEF promoter specific transcription is present in normal human keratinocytes

To determine whether increased nuclear levels of TCF/LEF–catenin complexes could affect the level of transcription from genes containing TCF/LEF response elements, keratinocytes were transiently transfected with TCF/LEF reporter constructs containing an optimized TCF binding site (Topflash) or a mutant TCF binding site (Fopflash) upstream of the luciferase gene (Fig 5). For normal human keratinocytes, luciferase activity was approximately an order of magnitude lower in keratinocytes transfected with the Topflash reporter than in those transfected with Fopflash (Fig 5a). This is the reverse situation from what has been observed in cells in which Wnt signaling is active. For example, because SW480 cells have high levels of cytoplasmic and nuclear β -catenin, the signaling function of β -catenin downstream of frizzled receptors is constitutively active; therefore, SW480 colorectal carcinoma cells serve as useful controls for this reporter assay. Consistent with published studies (Korinek *et al*, 1997; Morin *et al*, 1997), high levels of luciferase activity were detected in SW480 cells transfected with Topflash (Fig 5c); this activity was diminished 10-fold in SW480 cells transfected with the Fopflash construct containing the mutated TCF/LEF promoter sequence. The observation that luciferase activity in keratinocytes transfected with Topflash and Fopflash was the reverse of what was observed in SW480 cells suggests

the presence in human keratinocytes of a repressor capable of suppressing transcription from TCF/LEF optimized promoters, although we have not excluded the possibility that keratinocytes express an as yet unidentified transcriptional regulatory complex that preferentially binds Fopflash.

Negative regulation of gene transcription by TCF/LEF–plakoglobin complexes

When Topflash-transfected keratinocytes were treated with peroxovanadate, luciferase activity was essentially unchanged (Fig 5b, cols N, V). In contrast to β -catenin–TCF/LEF complexes, plakoglobin–TCF/LEF complexes do not efficiently bind DNA (Zhurinsky *et al*, 2000). The inability of plakoglobin–TCF/LEF to form high affinity ternary complexes with DNA suggested that one consequence of competition by plakoglobin with β -catenin for binding to TCF/LEF proteins might be downregulation of transcription from TCF/LEF responsive genes. Because transcription from TCF/LEF responsive promoters is repressed under the conditions of keratinocyte culture, additional transcriptional repression due to competition by plakoglobin for TCF/LEF might not be observed. To bypass these constraints, keratinocytes were incubated in the presence of LiCl prior to exposure to peroxovanadate. Consistent with the effects of LiCl in other cell types (Hedgepeth *et al*, 1997), increased luciferase activity was observed when keratinocytes were cultured in the presence of LiCl (Fig 5b, col L). Subsequent exposure of LiCl-

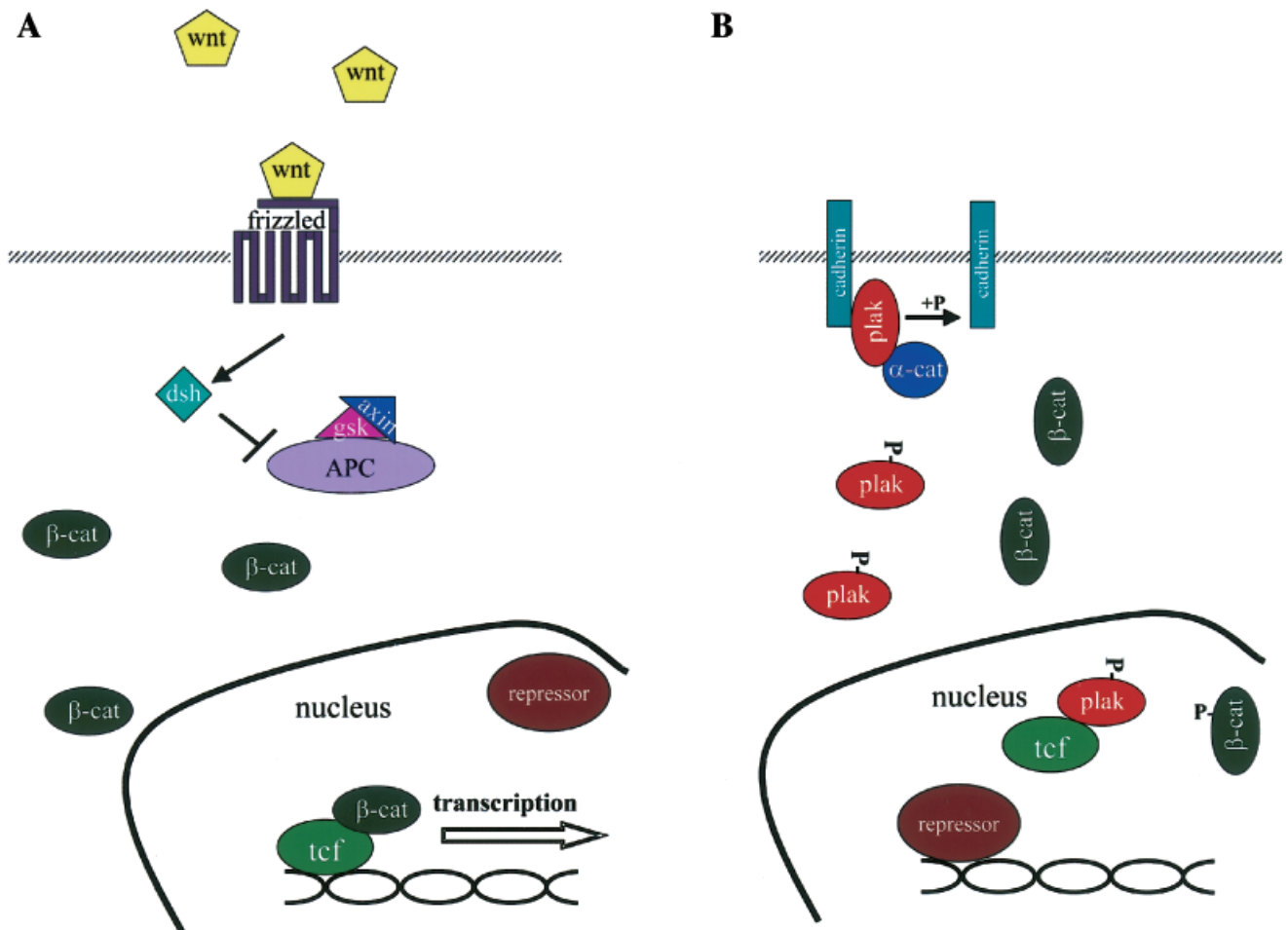


Figure 6. A model for adherens junction signaling. (A) Although β -catenin is constitutively synthesized, its cytoplasmic and nuclear levels are kept low by proteasome-mediated degradation through the targeting actions of a complex formed between APC, GSK-3, and axin. In the classic Wnt signaling pathway, the binding of Wnt proteins to frizzled transmembrane receptors initiates a signal that through the actions of dishevelled (dsh), suppresses APC/GSK-3/axin activity. As a result, cytoplasmic and nuclear β -catenin accumulate, driving formation of a transcriptionally active complex between β -catenin and TCF/LEF proteins. (B) Tyrosine phosphorylation of plakoglobin disrupts the adherens junction. Plakoglobin released from the adherens junction moves from the membrane to the cytoplasm and nucleus where it competes with β -catenin for binding to TCF/LEF proteins. The displacement of β -catenin from TCF/LEF proteins by plakoglobin forms transcriptionally inactive plakoglobin–TCF/LEF complexes resulting in functional antagonism of β -catenin-mediated Wnt signaling.

treated keratinocytes to peroxovanadate resulted in an approximately 4-fold decrease in luciferase activity (**Fig 5b**, col L + V).

Binding of Wnt/Wg proteins to frizzled cell surface receptors physiologically suppresses β -catenin degradation allowing cytoplasmic and nuclear levels of β -catenin to rise and activate transcription from TCF/LEF response elements. Incubation of keratinocytes with wingless-conditioned medium provided a second approach to activate downstream elements of Wnt signaling prior to disruption of the adherens junction. Like LiCl, incubation of keratinocytes with wingless-conditioned medium resulted in increased luciferase activity. When keratinocytes first incubated in the presence of wingless-conditioned medium were subsequently exposed to peroxovanadate, a reduction in luciferase activity was observed (**Fig 5b**, cols Wg, Wg + V) providing further support for plakoglobin's functional antagonism of β -catenin–TCF/LEF-mediated transcription.

Peroxovanadate is a potent inhibitor of tyrosine phosphatases and augments the tyrosine phosphorylation of many cellular proteins (Kadota *et al*, 1987; Ruff *et al*, 1997). Peroxovanadate-mediated changes in TCF/LEF reporter activity might be a consequence of nuclear translocated plakoglobin; alternatively, other molecular events might be responsible, including direct effects of peroxovanadate on β -catenin's binding to TCF/LEF, the interaction of β -catenin–TCF/LEF complexes with DNA, binding to

components of the basal transcription machinery, or transcription and/or translation of luciferase. Therefore, it was necessary to demonstrate that peroxovanadate was not affecting other cellular components that affect transcription from the Topflash reporter. Analogous to keratinocytes pretreated with LiCl or wingless, SW480 cells have elevated levels of cytoplasmic and nuclear β -catenin (Rubinfeld *et al*, 1996; Korinek *et al*, 1997) and serve as useful controls for activation of Wnt signaling components downstream of frizzled receptors. If the decrease in TCF/LEF reporter activity observed in keratinocytes were due to direct effects on β -catenin, TCF/LEF, or the transcriptional machinery, then treating SW480 cells with peroxovanadate should similarly result in decreased reporter activity. No significant difference in luciferase activity was observed between control and peroxovanadate-treated SW480 cells (**Fig 5d**), however, indicating that peroxovanadate-induced tyrosine phosphorylation does not directly affect components of Wnt signaling downstream of the cell surface.

DISCUSSION

Although the function of catenins in mediating Wnt signaling downstream of transmembrane frizzled receptors is well established, catenin signaling downstream of the adherens junction

has not previously been demonstrated. In human keratinocytes, we observed that peroxovanadate-mediated tyrosine phosphorylation of adherens-junction-associated plakoglobin resulted in (1) the rapid translocation of plakoglobin from cell-membrane-localized adherens junctions to the nucleus, (2) increased binding of plakoglobin to TCF/LEF proteins, and (3) inhibition of transcription from TCF/LEF optimized promoters. These observations support the concept of an additional signal transduction pathway initiated by changes in adherens junction structure that is mediated by redistribution of catenins from the adherens junction to the nucleus. In this pathway (**Fig 6**), disruption of β -catenin's and plakoglobin's association with E-cadherin and α -catenin, mediated by tyrosine phosphorylation, is followed by a rapid translocation of plakoglobin and β -catenin to the nucleus. For normal human keratinocytes in tissue culture, the effect of this translocation is a competition by plakoglobin with β -catenin for binding to TCF/LEF family members.

As a consequence of translocation of adherens-junction-associated plakoglobin to the nucleus, plakoglobin displaced β -catenin binding and/or trapped free TCF/LEF proteins, preventing the formation of functional transcriptional activation complexes with DNA. Although TCF/LEF- β -catenin complexes stimulate transcription after binding to TCF/LEF promoters, TCF/LEF-plakoglobin complexes do not bind directly to TCF/LEF promoters (Zhurinsky *et al*, 2000). Competition by plakoglobin with β -catenin for binding to TCF/LEF proteins may be the mechanism by which plakoglobin antagonized activation of transcription by β -catenin-TCF/LEF.

Signaling through the adherens junction may function to "inform" the cell of the changed state of adhesion, enabling cells to communicate and change their behavior depending on the presence or absence of cell-cell contact. In the absence of other signals, the effect of loss of cell adhesion by disruption of the adherens junction might be to block the transcriptional activation by β -catenin-TCF/LEF, thereby exerting an antiproliferative effect. Proliferation of nonadherent cells and the loss of contact inhibition often characterize malignant transformed cells. Thus, the antiproliferative signal mediated by plakoglobin released from the adherens junction might be one mechanism utilized by cells to prevent continued proliferation in the absence of adhesion. This would prevent proliferation of nonadherent cells as might occur in malignant tumors. As there are conditions in which continued proliferation of nonadherent or weakly adherent cells is of value, e.g., during growth and development and wound repair, other signals might impact on this pathway to regulate the balance of plakoglobin/ β -catenin binding to TCF/LEF transcription factors. β -Catenin-mediated signaling is observed during proliferative states; for example, stabilization of β -catenin and constitutive signaling has been observed in a variety of malignant tumors (Korinek *et al*, 1997; Morin *et al*, 1997; Rubinfeld *et al*, 1997; Fukuchi *et al*, 1998; Miyoshi *et al*, 1998; Voeller *et al*, 1998). Additionally, increased levels of cytoplasmic β -catenin staining have been identified in keratinocytes that give rise to the proliferative compartment of the epidermis (Zhu and Watt, 1999).

If the physiologic role of adherens junction signaling mediated by plakoglobin is to exert an antiproliferative effect, the loss of plakoglobin function might be associated with malignant transformation. This appears to be the case as (1) loss of heterozygosity in the plakoglobin gene has been reported in breast and ovarian tumors consistent with a putative tumor suppressor role for plakoglobin (Aberle *et al*, 1995), (2) reduced plakoglobin mRNA and protein levels have been identified in cervical carcinoma cell lines (Denk *et al*, 1997), (3) relative to weakly invasive cell lines, highly invasive breast cancer cell lines express decreased levels of plakoglobin (Sommers *et al*, 1994), (4) reduced plakoglobin expression correlates with decreased disease free intervals and lower survival rates in non-small cell lung cancer (Pantel *et al*, 1998), and (5) reduced expression of plakoglobin has been observed in pharyngeal squamous cell carcinoma (Pukkila *et al*, 2001). Furthermore, transfection of renal cell carcinoma cells with plakoglobin has been

shown to suppress their ability to form tumors in nude mice (Simcha *et al*, 1996). Consistent with an antiproliferative role for plakoglobin, overexpression of full length or N-terminally truncated plakoglobin in murine skin reduces epidermal proliferation and promotes apoptosis (Charpentier *et al*, 2000). Interestingly, we observed cleavage of PARP in nuclei of peroxovanadate-treated cells. PARP cleavage is a marker of apoptosis and may reflect one biologic consequence of plakoglobin's antagonism of β -catenin-mediated Wnt signaling. Despite these observations, in other contexts, plakoglobin has been reported to have oncogenic potential; Kolligs and coworkers have reported that plakoglobin may behave as an oncogene in an adenovirus E1A-transformed rat kidney epithelial cell line, possibly due to regulation of c-Myc expression (Kolligs *et al*, 2000). Therefore, the physiologic role of plakoglobin in activating or repressing signaling may be both cell and context dependent.

In summary, we have demonstrated that plakoglobin, released from the adherens junction, antagonizes β -catenin-mediated Wnt signaling, downregulating transcription from TCF/LEF response elements. The finding of reduced plakoglobin expression in a variety of tumors suggests that this signaling pathway is probably of physiologic significance and may function to antagonize proliferative signals when cell-cell adhesion is lost.

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